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Association of mitogen-activated protein kinase with the microtubule cytoskeleton

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ABSTRACT **Using indirect immunofluorescence microscopy and biochemical techniques, we have determined that approximately one-third of the total mitogen-activated protein kinase (MAPK) is associated with the microtubule cytoskeleton in NIH 3T3 mouse fibroblasts. This population of enzyme can be separated from the soluble form that is found distributed throughout the cytosol and is also present in the nucleus after mitogen stimulation. The microtubule-associated enzyme pool constitutes half of all detectable MAPK activity after mitogenic stimulation. These findings extend the known *in vivo* associations of MAPK with microtubules to include the entire microtubule cytoskeleton of proliferating cells, and they suggest that a direct association of MAPK with microtubules may be in part responsible for the observed correlations between MAPK activities and cytoskeletal alteration.**

Mitogen-activated protein kinase (MAPK), also known as the extracellular signal-regulated kinase (ERK), is involved in the transmission of signals between plasma membrane receptors and the nucleus (1, 2). MAPK has been shown to phosphorylate and regulate cytoskeletal components such as the microtubule-associated proteins *in vitro*, and it was originally named microtubule-associated protein-2 kinase after its substrate, MAP2 (3). Nonetheless, numerous immunocytochemical analyses have failed to show an association of MAPK with microtubules in proliferating cells (4–9). Thus, despite the often misunderstood nature of the original name, it is generally considered that MAPK is not actually associated with the microtubules in systems where mitogenesis occurs. However, MAPK was shown to associate with microtubules in certain rat brain dendrites *in situ* (10) and to copolymerize with bovine brain microtubules *in vitro* (11). MAPK was also shown to associate with the microtubule-organizing centers, but not spindle structures, in mouse oocytes (12). These findings raise the possibility that MAPK physically interacts with and regulates microtubule dynamics under certain unique circumstances such as meiosis and dendritic remodeling in the brain.

In proliferating cells, significant evidence suggests that MAPK plays a role in cytoskeletal regulation. In addition to MAPs found only in the brain, such as MAP2 and tau, MAPK also phosphorylates cytoskeletal components present in cycling cells such as MAP4 and caldesmon (13, 14). MAPs, which bind to and stabilize microtubules, are phosphorylated in response to cell stimulation by a variety of mitogens. The resulting phosphorylation inhibits their capacity to stabilize the microtubules (15). MAPK, which is activated by these mitogens, has been shown to be causative in MAP inhibition *in vitro* (13, 16). MAPK activation is triggered not only by a large number of mitogens but also upon integrin–extracellular matrix association (17, 18), a first step toward cell spreading. Although these findings collectively imply a possible role for MAPK in the regulation of the cytoskeleton, the above-described immunofluorescence evidence suggests that this regulation might be indirect.

In this report we examine the distribution of MAPK in cultured cells by using monoclonal antibodies (mAbs) directed against a variety of MAPK epitopes. We demonstrate that a physical association between MAPK and microtubules occurs in cycling cells and that this interaction can mask some of the MAPK epitopes. We further show that half of all cellular MAPK activity generated by mitogenic stimulation is microtubule-associated. These findings suggest that the regulation of cytoskeletal dynamics is an important aspect of the mitogenic response and that a significant proportion of MAPK activity is directed to microtubule-associated substrates outside the nucleus.

MATERIALS AND METHODS

Cell Culture and Preparation of Cytoskeleton. NIH 3T3 mouse fibroblasts were maintained in Dulbecco's modified Eagle's medium (DME) supplemented with 10% calf serum and grown at 37°C in a 5% CO_2/95% air atmosphere. For experiments involving stimulation by mitogens, cells were grown in DME supplemented with 0.1% calf serum for 16–18 hr. These "serum-starved" cells were then stimulated with calf serum (to 10%) or epidermal growth factor (50 ng/ml). Cytoskeleton was prepared as described (19) with the addition of phosphatase inhibitors (1 mM Na_3VO_4 and 1 μM microcystin LR) to the cytoskeleton-stabilizing buffer. For immunocytochemistry, cytoskeleton and nuclear matrix were methanol-fixed to glass coverslips after two extractions with cytoskeleton-stabilizing buffer containing 0.2% Triton X-100. For kinase and immunoblot assays, the two soluble fractions were pooled and supplemented with disodium β-glycerol phosphate (to 20 mM) and NaCl (to 150 mM). The remaining cytoskeleton and nuclear matrix were scraped off the plate into TBST (10 mM Tris, pH 7.5/150 mM NaCl/0.2% Triton X-100/10 mM EDTA/20 mM disodium β-glycerol phosphate/1 mM Na_3VO_4/1 μM microcystin LR containing leupeptin at 2 μg/ml, aprotinin at 1 μg/ml, and phenylmethanesulfonyl fluoride at 200 μg/ml) and chilled on ice for 1 min, mixed in a vortex mixer, and then centrifuged. The cytoskeleton fraction was removed and the nuclear matrix/intermediate filament pellet was solublized in 1 vol of 0.3% SDS and diluted in 9 vol of TBST. The three subcellular fractions were adjusted to equal volumes prior to further analysis.

Immunocytochemistry and Immunoblotting. Immunofluorescence staining of fixed cells and cytoskeleton was carried out as described (9) with the following exceptions. Paraformaldehyde-fixed cells were extracted with Triton X-100 prior to staining, except when the mAb anti-MAP kinase (Zymed) was

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Abbreviations: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MAP, microtubule-associated protein; mAb, monoclonal antibody; pAb, polyclonal antibody; NM/IF, nuclear matrix/intermediate filament.

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used, which required extraction with 0.1% SDS. For antibody competition, recombinant ERK2 (9) was boiled in 0.1% SDS and then diluted to a final concentration of 28 μg/ml in the antibody solution (final SDS concentration of 0.006%) and incubated for 1 hr at room temperature before application to the coverslips overnight at 4°C. For anti-pan ERK/vimentin costains, fluorescein isothiocyanate-conjugated rat anti-mouse IgM and biotin-conjugated rat anti-mouse IgG2a mAbs (Biosource International, Camerillo, CA) were used at 5 μg/ml as secondary antibodies, and tetramethylrhodamine-conjugated streptavidin (1 μg/ml; Molecular Probes) was applied for 30 min. Coverslips were mounted onto glass slides and observed under fluorescence by using a Nikon Diaphot-TMD inverted microscope equipped with a Plan Apo 60 lens. Immunoblots were prepared and analyzed as described (9), except that they were developed by using chemiluminescence (Renaissance kit; DuPont/NEN).

Anti-pan ERK (IgG2a), anti-ERK1 mAbs (Transduction Laboratories, Lexington, KY), and anti-MAP kinase mAb were used at 10 μg/ml for immunocytochemistry and 0.25 μg/ml for immunoblotting. Anti-vimentin (IgM) mAb (Sigma) was used at a dilution of 1:50 for immunocytochemistry and 1:1000 for immunoblotting. Rabbit anti-tubulin (20) and anti-actin (21) were generous gifts of J. Chlöe Bulinski (Columbia University, New York) and were used at 1:1000 and 1:2500, respectively, for immunocytochemistry and at 1:20,000 and 1:40,000, respectively, for immunoblotting.

MAPK Assays. MAPK activity analyses using myelin basic protein as the substrate and Mono Q (Pharmacia) ion-exchange chromatography were carried out as previously described (22) with slight modification. All buffers used were unchanged; however, a small amount of Triton X-100 (0.04% final concentration) was carried over from the samples and was also present (at 0.05%) during ion-exchange chromatography.

RESULTS

MAPK Costains with the Cytoskeleton. Indirect immunofluorescence was used to examine the subcellular localization of MAPK in NIH 3T3 cells (Fig. 1). Intact cells were fixed and treated with three different anti-MAPK mAbs (Fig. 1 *A*, *D*, and *G*). A MAPK–cytoskeleton association was clearly apparent with two out of three of the anti-MAPK mAbs [anti-pan ERK (Fig. 1 *A* and *B*) and anti-ERK1 (Fig. 1 *D* and *E*)]. These antibodies attached to curved cytoskeletal arrays that were immersed within a broader diffuse pattern of reactive material (Fig. 1 *A* and *D*). After the cells had been extracted to remove soluble proteins, cytoskeletal arrays maintained the associated MAPK staining (Fig. 1 *B* and *E*). Identical results were obtained when these antibodies were applied to Rat2 and TC7 cells (data not shown). The third mAb (anti-MAP kinase) had been used previously by others to examine the subcellular distribution of MAPK (5, 7). Consistent with these studies, this mAb reacted primarily with the diffuse cytoplasmic enzyme in the fixed cells (Fig. 1*G*) and showed no significant binding to prepared cytoskeleton (Fig. 1*H*). Similar results were obtained when a polyclonal antibody (pAb) recognizing the same epitope (residues 305–327 of ERK1) was used to stain cells (9) and prepared cytoskeleton (data not shown). For all three mAbs, preincubation with a 10-fold excess (mol/mol) of recombinant ERK2 abrogated the cytoskeletal immunofluo-

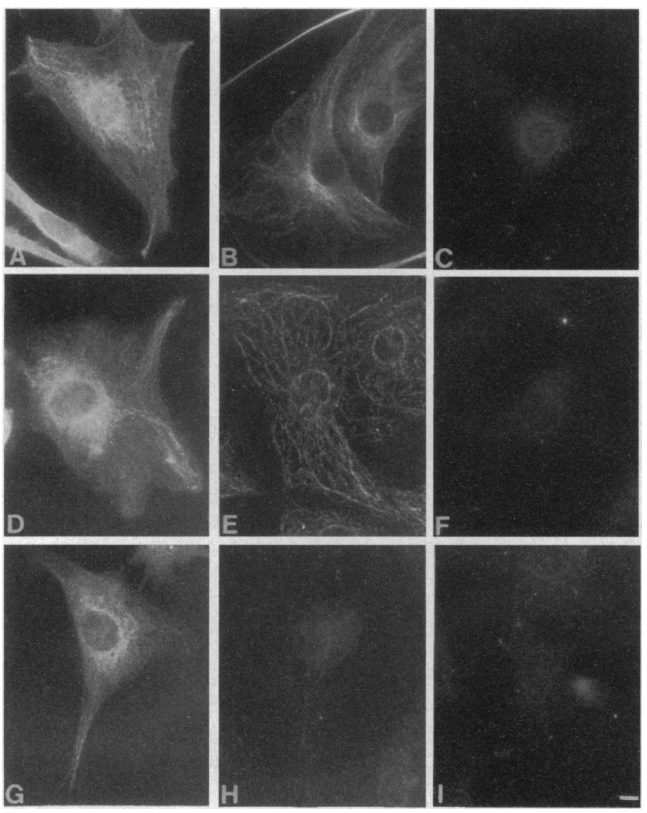

FIG. 1. Immunocytochemical staining of MAPK in NIH 3T3 cells. Cells (*A*, *D*, and *G*) and cell cytoskeleton (*B*, *C*, *E*, *F*, *H*, and *I*) were prepared and stained as described in the text. mAbs used for immunostaining were anti-pan ERK (*A–C*), anti-ERK1 (*D–F*), and anti-MAP kinase (*G–I*). In *C*, *F*, and *I*, recombinant ERK2 was added to the antibody mixes for 1 hr prior to the staining. [Bar (bottom right panel) = 10 μm.]

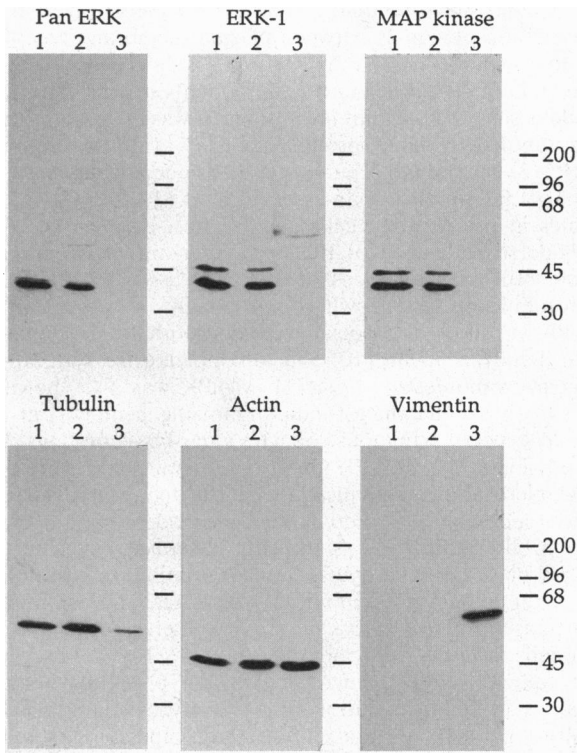

FIG. 2. Subcellular distribution of MAPK and cytoskeletal markers in soluble, cytoskeletal, and NM/IF fractions. NIH 3T3 cells were grown and then fractionated into soluble (lane 1), cytoskeletal (lane 2), and NM/IF (lane 3) pools and analyzed by immunoblotting. Membranes were probed with three anti-MAPK mAbs (*Upper*), including (from left to right) anti-pan ERK, anti-ERK1, and anti-MAP kinase, and with antibodies against cytoskeletal markers (*Lower*), including X^2 (anti-tubulin pAb), G^2 (anti-actin pAb), and anti-vimentin mAb. Positions for molecular mass standards (kDa) are marked to the right.

rescence (Fig. 1 *C*, *F*, and *I*), indicating that the patterns generated by these antibodies were MAPK specific.

MAPK Cofractionates with the Cytoskeleton. The above immunocytochemical analyses showed that epitopes recognized by two of three anti-MAPK mAbs are exposed on the cytoskeleton, while the epitope recognized by the third mAb is masked. Immunoblot analyses of soluble, cytoskeletal, and nuclear matrix/intermediate filament (NM/IF) fractions were then used to show that MAPK was present in the cytoskeletal fraction. Subcellular fractions were prepared and probed using the three anti-MAPK mAbs (Fig. 2 *Upper*) and antibodies against tubulin, actin, and vimentin (Fig. 2 *Lower*). These analyses showed that, irrespective of their designations, both the anti-ERK1 and the anti-MAP kinase mAbs bound to ERK1 (44 kDa) and to ERK2 (42 kDa), while the anti-pan ERK mAb bound predominantly to ERK2 (Fig. 2 *Upper*). All three of these mAbs showed the presence of both MAPK isoforms in the cytoskeletal fraction (Fig. 2 *Upper*, lane 2 of each gel). The anti-pan ERK mAb also bound very weakly to a 54-kDa protein once described as a MAP-2 kinase, but now known as the stress-activated protein kinase (23), which was predominantly found in the cytoskeletal fraction (lane 2). ERK1 and ERK2 were also present in the soluble fraction (Fig. 2 *Upper*, lane 1 of each gel) but absent from the NM/IF fraction (lane 3). Densitometric analyses of the MAPK in the immunoblots showed that 35% of ERK1 and 42% of ERK2 were present in the cytoskeleton-bound pool of these cells. These analyses indicated that the anti-MAPK mAbs bound specifically to MAPK isoforms in the cytoskeletal fractions and that the epitope for the third mAb is masked when MAPK is in a cytoskeleton-bound form (as shown in Fig. 1*H*). Identical analyses using the probes for the cytoskeletal markers (Fig. 2 *Lower*) revealed that tubulin, like MAPK, was largely restricted to the soluble (lane 1) and cytoskeletal (lane 2) fractions with only a trace present in the NM/IF fraction (lane 3). Although actin was present in both the soluble and cytoskeletal fractions (lanes 1 and 2), it was also significantly associated with the NM/IF fraction (lane 3), in which almost all of the vimentin resides.

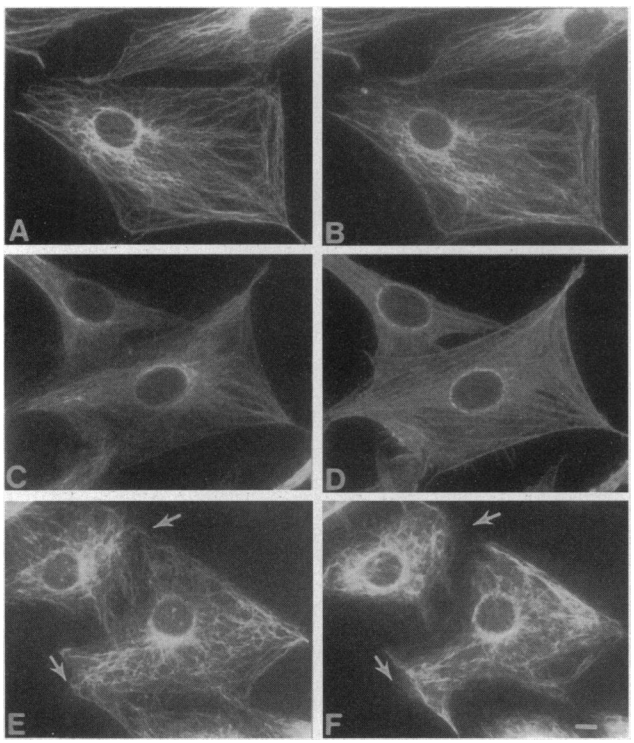

FIG. 3. Colocalization of MAPK with microtubules. Cell cytoskeleton from serum-starved NIH 3T3 cells was prepared and stained. MAPK was visualized by using the anti-pan ERK (IgG2a) mAb (*A*, *C*, and *E*), and cytoskeleton markers were detected with the following: X^2 (anti-tubulin) pAb (*B*), G^2 (anti-actin) pAb (*D*), and the anti-vimentin (IgM) mAb (*F*). (Bar = 10 μm.)

MAPK Is Distributed Along Microtubules. To directly compare the MAPK immunofluorescence pattern to that of cytoskeletal markers, double staining of prepared cytoskeleton was carried out (Fig. 3). The immunofluorescence pattern generated by the anti-pan ERK (Fig. 3*A*) and anti-ERK1 mAbs precisely matched that of the anti-tubulin serum (Fig. 3*B*). This alignment can be extended to include cells undergoing mitosis or cytokinesis. Pretreatment of the cells with nocodazole or exposure to cold disrupted both the MAPK and tubulin patterns without significantly disrupting the patterns for the other two cytoskeletal networks (data not shown). In preparations costained with actin antisera, MAPK displayed some coincidence with actin at the cell periphery where stress fibers and microtubules overlap (Fig. 3 *C* and *D*). Treatment of the cells with cytochalasin D disrupted the actin cytoskeleton, but not the MAPK or tubulin patterns (data not shown). Costaining of MAPK and vimentin (Fig. 3 *E* and *F*) indicated that these proteins overlapped. However, unlike the staining of MAPK and tubulin, that of vimentin often failed to extend to the cell periphery (Fig. 3*F*, arrows); it was less finely distributed throughout the cytoplasm and largely insensitive to nocodazole treatment. Together, these data suggest that MAPK is distributed along microtubules rather than along actin stress fibers or intermediate filaments.

Half of the Mitogen-Stimulated MAPK Activity Is Microtubule-Associated. Initial immunocytochemical analyses showed that microtubule-bound MAPK could be separated from the diffuse enzyme upon extraction of adherent cells (shown in Fig. 1). It was further demonstrated that the enzyme found in the nucleus after mitogen stimulation could also be separated from the microtubule-bound pool (Fig. 4). Control serum-starved cells and cells stimulated for 30 min with calf serum were stained with the anti-pan ERK mAb. As expected,

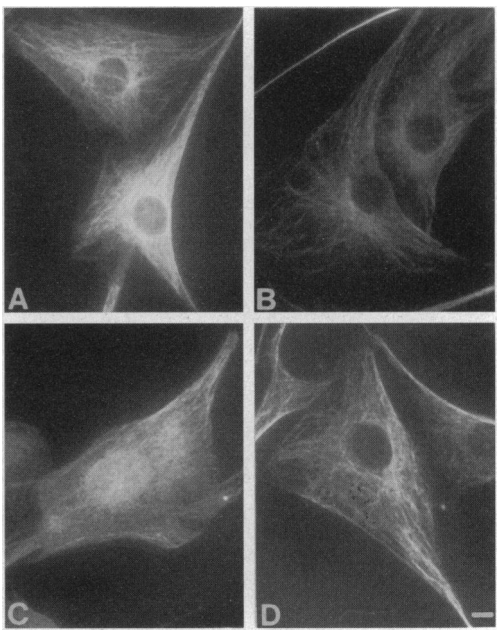

FIG. 4. Microtubule-associated MAPK can be separated from the nuclear and cytosolic enzyme. Serum-starved cells (*A* and *B*) and cells stimulated with serum for 30 min (*C* and *D*) were either fixed (*A* and *C*) or fixed as cytoskeleton after soluble proteins were extracted (*B* and *D*). Samples were then stained with the anti-pan ERK mAb as described for Fig. 1. Note that the nuclear matrix (*D*), unlike the intact nucleus (*C*), of stimulated cells is devoid of significant MAPK staining. (Bar = 10 μm.)

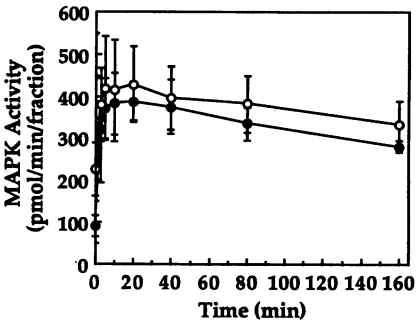

FIG. 5. Comparison of soluble and cytoskeletal MAPK activity after serum stimulation. G_0 cells were stimulated for 0–160 min with 10% serum. Cytoskeletal (○) and soluble (●) fractions from 1 mg of NIH 3T3 cellular protein were assayed for MAPK activity using myelin basic protein as a substrate. The total amount of measurable activity (pmol of phosphate transferred per min) in each fraction is plotted against the time of stimulation.

no significant amount of MAPK was found in the nuclei (Fig. 4*A*) or in the nuclear matrices attached to the cytoskeleton of unstimulated cells (Fig. 4*B*). By contrast, MAPK was observed to translocate to the nuclei after serum stimulation (Fig. 4*C*). The extraction procedure used to isolate the cytoskeleton and the nuclear matrix removed the nuclear and cytosolic MAPK, leaving only the microtubule-bound portion intact (Fig. 4*D*). Immunoblot analyses confirmed the absence of MAPK in the NM/IF fraction over a time course of 24 hr and showed that the average size of the cytoskeleton-bound pool remained unchanged during the first 4 hr of stimulation (data not shown).

Cells were next fractionated after serum stimulations of 0–160 min and the levels of MAPK activity present in the microtubule-bound and soluble pools were determined (Fig. 5). At all times, the MAPK activity was identical or slightly higher in the cytoskeletal pool than in the soluble fraction. When these were further fractionated (Fig. 6 *C* and *D*), two separate peaks of MAPK activity were identified in fractions 11–19 (peak 1) and 25–29 (peak 2). Immunoblot analyses (Fig. 6*E*), using anti-MAPK (*Upper*) and anti-phosphotyrosine (*Lower*) antibodies, indicated that peaks 1 and 2 were tyrosine-phosphorylated ERK2 and ERK1, respectively. Integration of the peaks after subtraction of the background showed that, after 10-min serum stimulation (Fig. 6*C*), MAPK activity was induced 10-fold in the cytoskeletal fraction and 7-fold in the soluble pool. The induced MAPK activities in both pools gradually declined throughout the entire time course of the reaction. After 160 min of serum stimulation, they remained 7-fold (cytoskeletal) and 4-fold (soluble) above background (Fig. 6*D*). When epidermal growth factor was used as a mitogen instead of serum, MAPK activity in both pools rapidly returned to basal levels (in ≈40 min). This shorter duration of epidermal growth factor-stimulated MAPK activity is consistent with previous findings from this laboratory (9, 22).

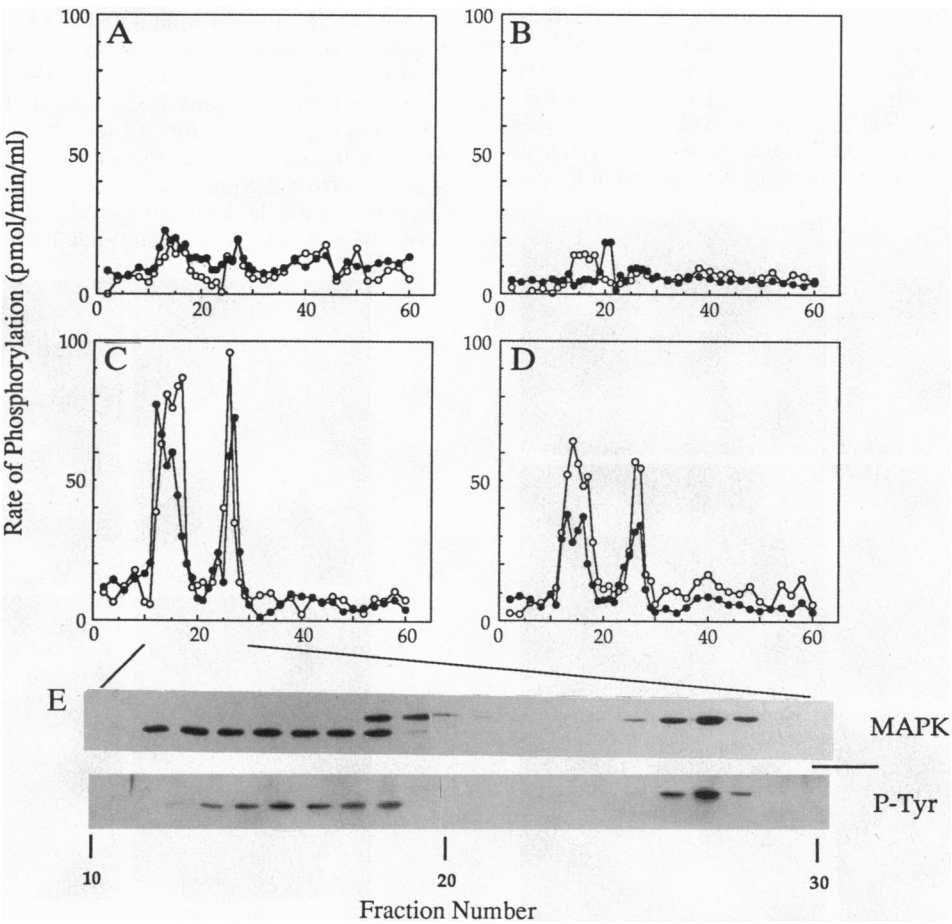

FIG. 6. Comparison of soluble and cytoskeletal MAPK activity after serum stimulation and subfractionation by Mono Q FPLC (Pharmacia). Soluble and cytoskeletal pools from 2 mg of NIH 3T3 cellular protein were subfractionated by Mono Q FPLC and assayed. Chromatograms display cytoskeletal (○) and soluble (●) MAPK activities and include the following: serum-starved cells (*A*), cycling cells (grown continuously in 10% serum; *B*), and cells stimulated with serum (10%) for 10 min (*C*) and 160 min (*D*). MAPK activities (pmol/min/ml) are plotted against the Mono Q fraction numbers indicated on the *x*-axes. The presence of active MAPK isoforms in the two activity peaks was identified by immunoblotting fractions 10–30 from the cytoskeletal fraction of cells stimulated for 10 min (*C*), using the 7884 (anti-MAPK) pAb (*E Upper*) and anti-phosphotyrosine mAb (*E Lower*).

DISCUSSION

It has been well documented that MAPK exists as a cytoplasmic enzyme that, upon stimulation, translocates in part to the nucleus. This report demonstrates that, in addition, a sizable portion of the enzyme is associated with microtubules in NIH 3T3 and other cultured cells. Anti-MAPK immunofluorescence was blocked by the addition of recombinant ERK2, demonstrating the specificity of the antibodies used. Furthermore, MAPK was found to cofractionate with the cytoskeleton. In defining the MAPK–microtubule association, preparations of intact and disrupted cytoskeleton were used; they show a perfect alignment of MAPK with the microtubules but not with actin or intermediate filaments. Together, these data clearly demonstrate that MAPK is associated with the microtubule cytoskeleton.

These results are consistent with evidence obtained *in situ* pointing to a similar type of association in certain nonproliferating brain cells (10). They contrast with immunofluorescence data indicating that MAPK is not microtubule-associated in cycling cells (4–9). In these latter reports which, incidentally, characterized the nuclear translocation of MAPK after mitogenic stimulation, the MAPK antibodies appeared to bind only to the free enzyme. That possibility was checked by using antibodies that recognized epitopes between residues 305 and 327 (Figs. 1 and 2) and between residues 352 and 367 (data not shown) of ERK1 (4–7, 9). These epitopes are indeed masked upon MAPK–microtubule interaction. They also are partially recognized after pretreatment of fixed samples with SDS. By contrast, the anti-ERK1 mAb, which recognizes a different epitope (residues 325–345) situated between these two previous ones, readily binds to the microtubule-associated MAPK.

It can be assumed that the active microtubule-bound MAPK is implicated in the regulation of microtubule dynamics. MAPK is known to phosphorylate the MAPs, thus having a negative impact on microtubule stability *in vitro* (as described above). The juxtaposition of both MAPK and the MAPs on the microtubules *in vivo* suggests a direct interaction between the two and that regulation may occur at these sites. Significant evidence also shows a correlation between MAPK activity and the organization of the cytoskeleton. The enzyme is activated when the cytoskeleton is rearranged during cellular responses to chemotactic agents in the absence of mitogenesis (24) or during neurite outgrowth in PC12D cells when transcription is blocked (25). MAPK is also activated during oncogenesis (26), which causes significant changes in microtubule organization (27). Oncogenesis can be achieved with the expression of constitutively active MAPK kinase-1 mutants (28, 29). Cells expressing these or other mutant forms (9) indicated that activation of MAPK correlates with a rounding up of cells, while inactivation of the enzyme correlates with their flattening. These results are consistent with others showing that a MAPK phosphatase inactivates MAPK and causes an enhanced spreading of the transfected cells (30, 31). Finally, both MAPK and the MAPs (32) associate with the microtubules that form the mitotic spindle and the cytokinetic midbody, suggesting that the kinase regulates microtubule dynamics during chromatin separation and cell division. Similar mechanisms may operate during meiosis in which microtubule dynamics correlate with the activation of MAPK rather than with that of the cyclin-dependent kinase that functions as the maturation-promoting factor (33).

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